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14. ABSTRACT
The main focus of the work was to explore engineered PRINT particles to elicit an immunological response. The primary objective was to test the idea that engineered PRINT particles could facilitate different T and B cell immune responses. A secondary objective was to use PRINT to make particles from biological and man-made master templates to afford particles of controlled shape down to <1 nm resolution and functionality. Molecular templating agents and viral-envelope proteins were investigated to augment the shape-specificity of our molded nanoparticles.

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Report Title

Final Technical Report: Replicating Viral Particles and other Shape-Controlled, Functional Particles for Targeted Delivery Applications Using Nano-Molding Techniques

ABSTRACT

The main focus of the work was to explore engineered PRINT particles to elicit an immunological response. The primary objective was to test the idea that engineered PRINT particles could facilitate different T and B cell immune responses. A secondary objective was to use PRINT to make particles from biological and man-made master templates to afford particles of controlled shape down to <1 nm resolution and functionality. Molecular templating agents and viral-envelope proteins were investigated to augment the shape-specificity of our molded nanoparticles.

Several strategies for conjugation a wide variety of ligands to the surface of the PRINT particles, including strategies for the binding of nitrophenyl, pneumococcal polysaccharide-C and streptavidin-biotin conjugates were developed. A major advantage of PRINT delivery is the ability to target the cargo to professional antigen presenting cells. Specific and effective targeting to dendritic cells was observed. Also explored was the particle uptake and release of cargo in vitro. PRINT particles have been fabricated that are comprised of disulfide cross-linkers that are sensitive to the reducing environment which allows them to dissolve in cellular vesicles. The results and progress over the period of the grant July, 2006 to July, 2007 are described.

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List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Maynor, B.W.; LaRue, I.; Rolland, J.P.; Spontak, R.J. Sheiko, S.S.; Samulski, R.J.; Samulski, E.T.; DeSimone, J.M. “Molecular Nanomimetics: Replication of Viruses, Micelles and other Naturally-Occurring Objects” submitted to Small 3 (2007) 845.

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(c) Presentations

1. Petros, Robby A.; Buntzman, Adam; Frelinger, Jeffrey A.; DeSimone, Joseph M. "PRINT nanoparticle-based cancer immunotherapies." Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19-23, 2007, COLL-106.

2. Petros, Robby A.; DeSimone, Joseph M. "PRINT: Nanomaterials for biological applications." Abstracts of Papers, 234th ACS National Meeting, Boston, MA, United States, August 19-23, 2007, AEI-070.

3. Petros, Robby A.; Buntzman, Adam; Euliss, Larken E.; DuPont, Julie A.; Frelinger, Jeffrey A.; DeSimone, Joseph, M. "Antibody conjugation to PRINT nanoparticles as a cellular targeting strategy." Abstracts of Papers, 233rd ACS National Meeting, Chicago, IL, United States, March 25-29, 2007, COLL-014.

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

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Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Number of Inventions:

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Adam Buntzmann	0.50
Rob Petros	0.50
FTE Equivalent:	**1.00**
Total Number:	**2**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Joseph DeSimone	0.05	Yes
Sergei Sheiko	0.05	No
Jeff Frelinger	0.05	No
Jude Samulski	0.05	No
FTE Equivalent:	**0.20**	
Total Number:	**4**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:...... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

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Final Technical Report

Replicating Viral Particles and Other Shape-Controlled, Functional Particles for Targeted Delivery Applications Using Nano-molding Techniques:
Full Proposal Submitted to DARPA
by

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Institute for Advanced Materials, Nanoscience and Technology[5]
Gene Therapy Center[6]
University of North Carolina at Chapel Hill[1-3,5,6]
North Carolina State University[4]

Below is the final technical report for the progress on the milestones associated with our program on **"Replicating Viral Particles and Other Shape-Controlled, Functional Particles for Targeted Delivery Applications Using Nano-molding Techniques"**.

Abstract:

The main focus of the work was to explore engineered PRINT particles to elicit an immunological response. The primary objective was to test the idea that engineered PRINT particles could facilitate different T and B cell immune responses. A secondary objective was to use PRINT to make particles from biological and man-made master templates to afford particles of controlled shape down to <1 nm resolution and functionality. Molecular templating agents and viral-envelope proteins were investigated to augment the shape-specificity of our molded nanoparticles.

Several strategies for conjugation a wide variety of ligands to the surface of the PRINT particles, including strategies for the binding of nitrophenyl, pneumococcal polysaccharide-C and streptavidin-biotin conjugates were developed. A major advantage of PRINT delivery is the ability to target the cargo to professional antigen presenting cells. Specific and effective targeting to dendritic cells was observed. Also explored was the particle uptake and release of cargo in vitro. PRINT particles have been fabricated that are comprised of disulfide cross-linkers that are sensitive to the reducing environment which allows them to dissolve in cellular vesicles. The results and progress over the period of the grant July, 2006 to July, 2007 are described.

<u>Milestone #1</u>

We will design PRINT particles which are decorated with pneumococcal polysaccharide-C (Pnp) to test T independent antibody responses. The particles will be injected into mice and their serum antibody responses will be measured. Efficacy will be compared to soluble Pnp.

Jeff Frelinger and Joseph DeSimone

We have designed PRINT particles that can be decorated with pneumococcal polysaccharide-C (Pnp). A general surface coupling strategy was developed, which utilizes carbonyldiimidazole as the coupling reagent. The hydroxyl group of PEG_{485}-monomethylmethacrylate can be reacted either pre- or post-PRINTing with carbonyldiimidazole to form a carbonyldiimidazole-modified PEG_{485}-monomethacrylate (see **Figure 1**). Virtually any moiety containing a nucleophilic species can then be covalently attached to PRINT particles. Particles were synthesized using preformed carbonylimidazole monomer with the following composition: PEG_{428}-triacrylate (39 wt%), PEG_{485}-carbonylimidazole-monomethacrylate (60 wt%), and 2,2-diethoxyacetophenone (1 wt%). SEM images are shown in **Figure 2**. The particles were harvested in DMSO, reacted with the antigen in solution and purified.

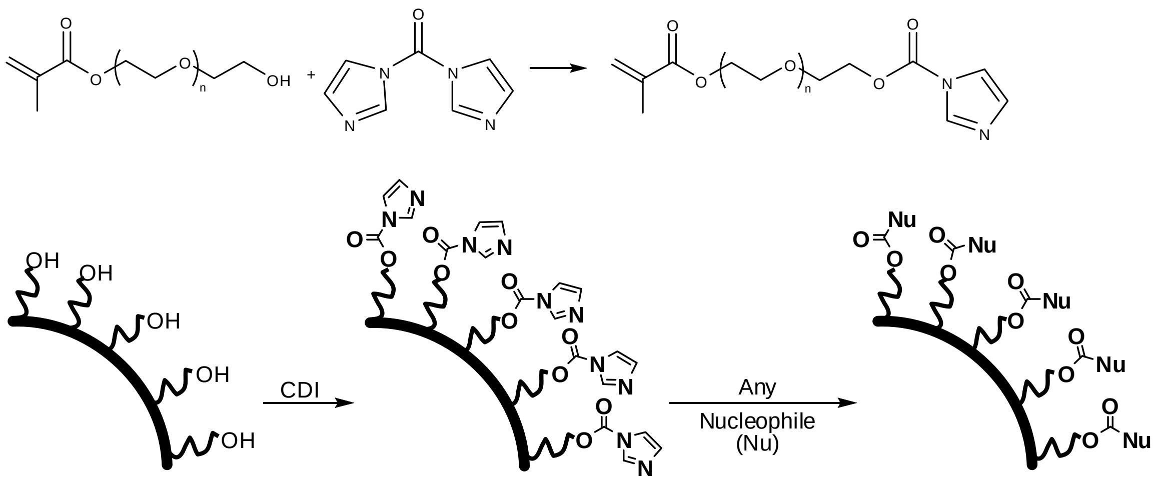


Figure 1 – Schematic depiction of the process for attaching targeting ligands to the surface of PRINT particles. Top – pre-PRINT strategy where PEG_{485}-monomethacrylate is reacted with CDI prior to PRINTing. Bottom – post-PRINT strategy where PEG_{485}-monomethacrylate is incorporated into the particle and then reacted with CDI.

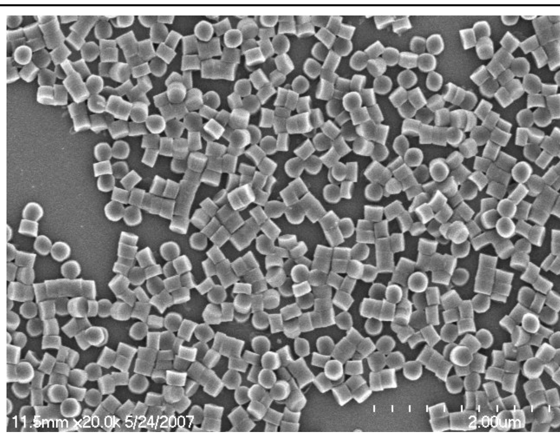


Figure 2 – SEM images of cylindrical PRINT particles (200 nm diameter × 200 nm height) that have a carbonylimidazole functionalized surface.

The pneumococcal polysaccharide type 5 (American Type Culture Collection), which is known to have one repeating unit containing a primary amine[1] was covalently coupled to the nanoparticle using the primary amine group following the strategy outlined above. Pnp is polymeric (~50 repeat units) and it is unlikely that all amino groups will be bound to particles (see **Figure 3**).

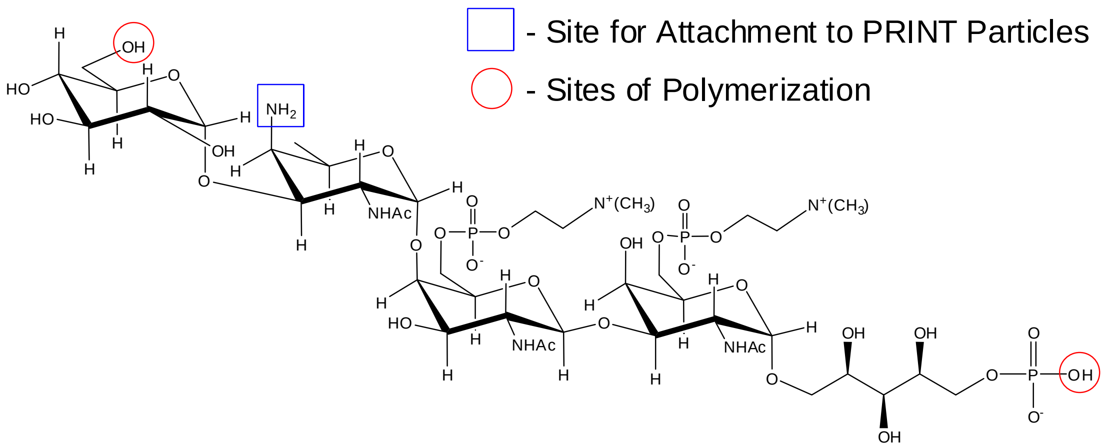


Figure 3 – Structure of pneumococcal polysaccharide type 5 showing both the sites of polymerization and the site for covalent attachment to PRINT particles.

The antibody responses of PRINT particles decorated with pneumococcal polysaccharide-C (Pnp) was tested. 150 microliters of PnP-PRINT particles in phosphate buffered saline (PBS) was injected into C57BL6 mice by the intraperitoneal route. The mice did not appear to experience any discomfort and no other phenotype was noted in the mice during the course of the experiment. At 7 days and 21 days post inoculation, approximately 150 microliters of peripheral blood was withdrawn from each mouse, via a submandibular puncture. The blood was allowed to clot, and the serum was isolated.

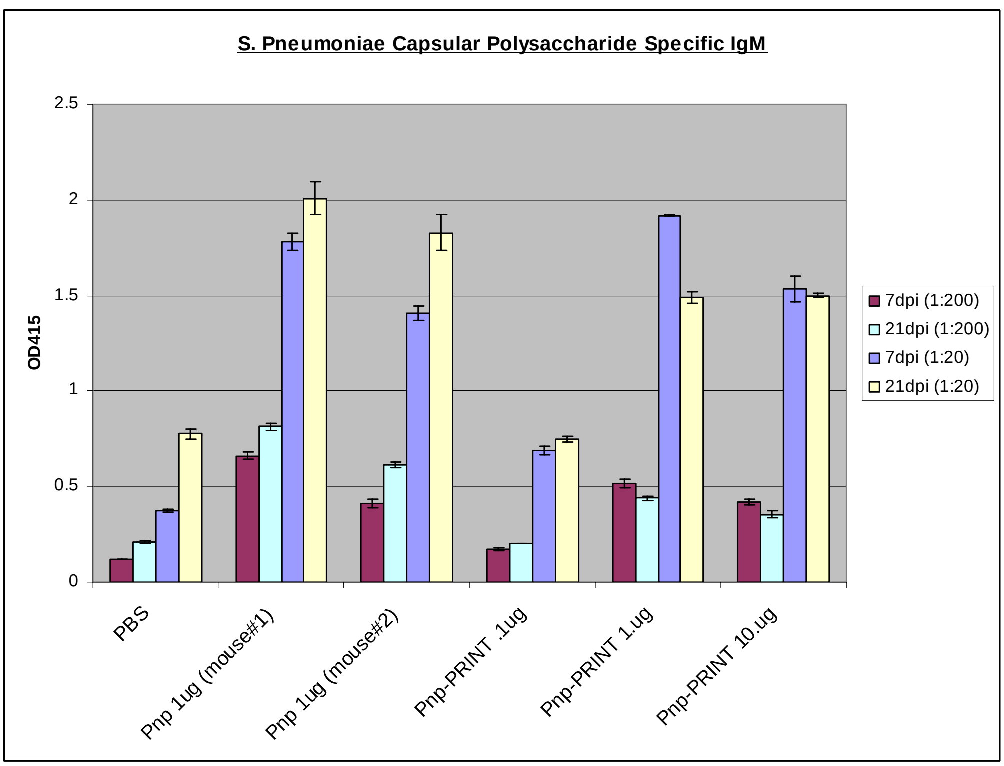


Figure 4 – Absorbance at 415 nm measuring the antibody (IgM) response of Pnp decorated PRINT particles. A pneumococcal polysaccharide specific ELISA was used. Serum collected at 7 and 21 days post inoculation. Positive controls were mice injected with free Pnp at a 1 μg concentration. Negative controls were mice injected with PBS. Serum was diluted by 1:20 or by 1:200 prior to being added to the ELISA plate. PnP decorated PRINT nanoparticles successfully elicited an immune response.

A pneumococcal polysaccharide specific ELISA was performed by binding the polysaccharide to an ELISA plate and then incubating the experimental serum on those polysaccharide plates to trap any polysaccharide specific antibodies that may have been generated by the mouse. Then the plates were exposed to a secondary antibody-HRP that detects either mouse IgM or mouse IgG. The plates were incubated with the HRP substrate and quantified spectrophotometrically. The absorbance at 415 nm for IgM is shown in **Figure 4** and in **Figure 5** for IgG. A mouse injected with phosphate buffered saline only acted as the negative control. The positive controls were mice that had been injected with 1 μg free Pnp which was not conjugated to a PRINT nanoparticle. The data points correspond to the 7 and 21 days post inoculation derived serum (7dpi and 20dpi). The serum was diluted 1:20 or 1:200 prior to being added to the ELISA plate.

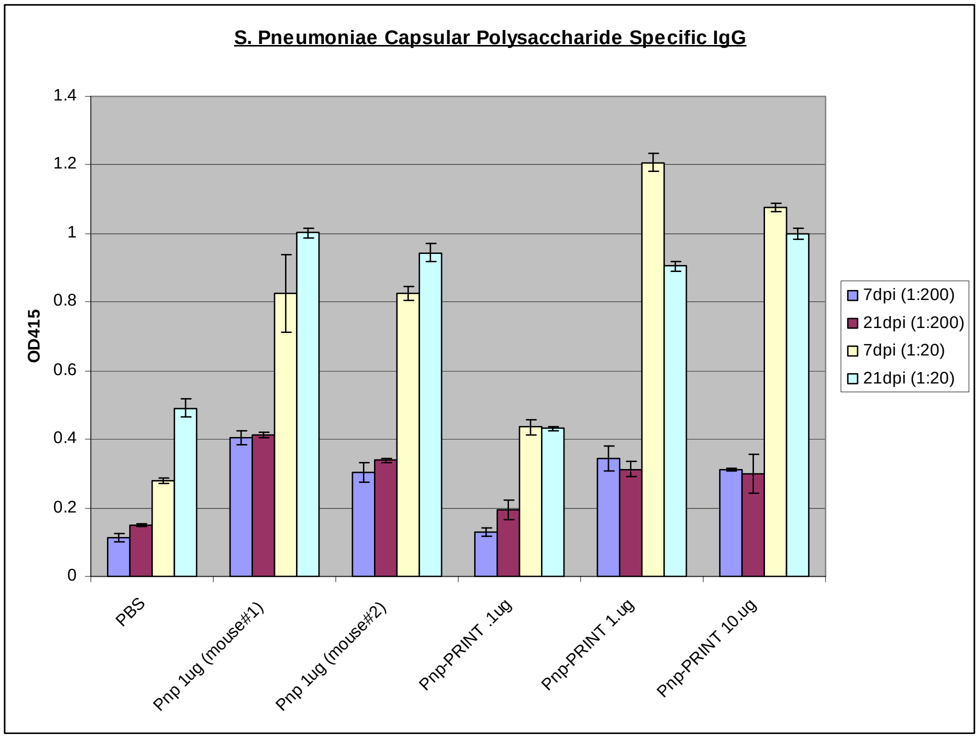


Figure 5 - Absorbance at 415 nm measuring the antibody (IgG) response of Pnp decorated PRINT particles. A pneumococcal polysaccharide specific ELISA was used. Serum collected at 7 and 21 days post inoculation. Positive controls were mice injected with free Pnp at 1 μg concentration. Negative controls were mice injected with PBS. Serum was diluted by 1:20 or by 1:200 prior to being added to the ELISA plate. PnP decorated PRINT nanoparticles successfully elicited an antibody response.

The Pnp decorated nanoparticles successfully elicited a T-cell independent antibody response that was on the order of the free Pnp. In addition, the ligand conjugated to PRINT particles are not toxic to B6 mice, don't interfere with a PnP induced humeral response and don't sequester themselves in a compartment that is inaccessible to whatever cell type is taking up this antigen and presenting it to the B cells.

The potential advantages of using the PRINT nanoparticles are ease of manufacturing due to the fact that each of the polysaccharides would not need to be individually conjugated. It is also advantageous to be able to sequester our different polysaccharides on the same particle. Encapsulation in a PRINT particle may also enhance the stability of conjugate vaccines due to the protein component conjugate vaccines.

Milestone #2

We will engineer PRINT particles decorated with nitrophenyl in order to test the ability of PRINT particles to stimulate B cells directly. In this case, we will add the particles to nitrophenyl specific splenic B cells derived from the B cell receptor transgenic mouse and measure the Ig production as a function of nanoparticle dose and ligand density.

Jeff Frelinger and Joseph DeSimone

In order to effectively conjugate nitrophenyl onto the surface of PRINT particles, we needed to develop conjugation chemistries so as to functionalize the PRINT particles with targeting ligands and small molecules. We have developed a second strategy for surface modifying PRINT particles based on installing reactive amine handles on the surface. We have prepared particles with as much as 11 wt% aminoethylmethacrylate incorporated (see **Figure 6**). We have reacted those amine groups on the surface with 2,4,6-trinitrobenzene 1-sulfonic acid (TNBS) which produced surface conjugated tri-nitrophenyl groups (TNP) (see **Figure 6**).[2] Initial studies showed a marked color change upon stirring a solution containing amine-functionalized PRINT particles with TNBS (**Figure 7**).

NP (nitrophenol)-decorated PRINT particles (conjugated to the surface as outlined in **Figure 6**) was added to purified NP specific splenic B cells derived from the B cell receptor transgenic mouse, B1-8.[3] The transgenic mouse allows a greater sensitivity for response. Ig production in those cultures will be assayed as a function of nanoparticle dose and ligand density. Our expectation was that the antigen bearing PRINT particles will stimulate a direct TI-2 response resulting in IgM production directed at the antigen.

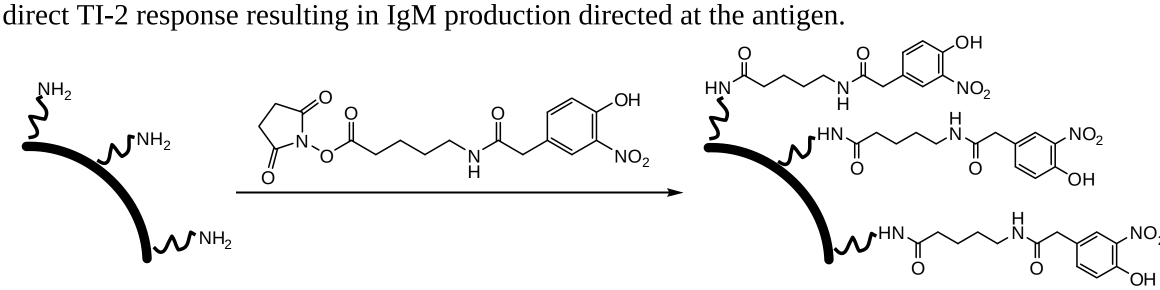


Figure 6 – Proposed method for conjugating nitrophenol (NP) groups to amine-functionalized PRINT particles.

Figure 7 – Solutions containing TNPS (left) and TNPS + PRINT particles (right) in PBS.

NOTE: The issues surrounding the MTA agreement with MGC Foundation, Mouse Genetics Cologne Foundation were not able to be resolved. Instead of the splenic B cells derived from the transgenic mouse B1-8, we will use the nitrophenol responsive B-cell hybridoma cells, B1-8 for the in vitro response.[4] These experiments will be completed when the cell lines arrive in house.

<u>Milestone #3</u>

To measure T cell responses *in vivo*, we will synthesize PRINT particles with an antibody specific for DEC 205 linked to the surface. By measuring the proliferation of $CD4^+$ and $CD8^+$ transgenic T cells, we can detect the T cell activation by dendritic cells. This will measure in vivo targeting.

Jeff Frelinger and Joseph DeSimone

DEC 205 has recently been shown to be expressed only on a subset of dendritic cells.[5] For the targeting to be as effective as possible we therefore switched to targeting CD11c instead of targeting DEC 205 since CD11c is expressed on all dendritic cells. This change will allow better targeting to the critical cells that regulate the afferent immune responses through their interaction with T cells. Using the CD11c we have been able to demonstrate selective targeting in vitro to CD11c splenic dendritic cells.

We decided that a major advantage of PRINT delivery was the ability to target the cargo to professional antigen presenting cells. As a result we spent our initial effort in improving the PRINT targeting technology in two ways. First was to alter the particle surface to decrease the non-specific binding. The initial PRINT particles used showed high levels of non-targeted uptake/binding by nearly all cells. Experiments to block the non-targeted binding ultimately revealed that unreacted functional groups on the PRINT surface resulted in covalent attachment to cell surfaces. This non-specific binding could be largely eliminated by treating the PRINT particles with ethanolamine following avidin coupling. The second was to determine the optimum concentration of the streptavidin coupling to the surface of the PRINT particle to allow control of the antibodies used for targeting. We have measured the concentration of streptavidin on the surface on PRINT particles by titration with biotin-4-fluorescein.[6,7] As biotin-4-fluorescein is titrated into a solution containing streptavidin it quickly binds altering the fluorescence intensity of the fluorescein tag. When binding is to free streptavidin in solution fluorescence is quenched; however, when the streptavidin is bound to PRINT particles more complicated behavior is observed. This phenomenon is not completely understood, but the response eventually becomes linear in both cases, which can be taken as an indication that all available binding sites have been filled. Changes are also observed in the fluorescence intensity of the Alexa Fluor 647 tag on streptavidin upon binding of biotin. Specifically, a PRINT particle solution was titrated with biotin-4-fluorescein while monitoring the fluorescence of both the fluorescein tag and the Alexa Fluor 647 tag on streptavidin (see **Figure 8**). The fluorescence of the fluorescein tag begins to increase linearly after the addition of approximately 90 μL of biotin. At the same time, the fluorescence of the Alexa Fluor 647 tag stabilizes. These two pieces of data indicate that all available binding sites have been filled after the addition of 90 μL of biotin. Assuming the mass of a single cylindrical PRINT particle (200 nm d × 200 nm h) to be 3.35×10^{-15} g, there were ~1240 biotin binding sites per particle. If it is further assumed that there are three available binding sites per copy of streptavidin (one binding site is disrupted by attachment to the particle) there were ~412 copies of streptavidin per particle.

The amount of biotin rat IgG2b bound to the surface of PRINT particles was measured by titration of a streptavidinated PRINT particle solution with biotin rat IgG2b isotype control. This assay takes advantage of the knowledge gained from the previous experiment where we determined the number of copies of streptavidin per PRINT particle. We learned that biotin binding, in addition to affecting the fluorescence of biotin-4-fluorescein, affects the fluorescence intensity of the Alexa Fluor 647 tag on streptavidin. A PRINT particle solution was titrated with a stock antibody solution while monitoring the fluorescence of the Alexa Fluor 647 tag on streptavidin (see **Figure 8**). Again, behavior similar to that of the biotin-4-fluorescein titration was observed for the Alexa tag. The fluorescence signal increased in intensity as the biotinylated antibody was added initially, and at certain point the readings began to stabilize as additional antibody was added. It was assumed that all accessible binding sites were filled when the readings began to stabilize. Based on a molecular weight of 150 kDa for the antibody, this corresponds to ~380 copies of the antibody per particle. This number correlates well with the number of copies of streptavidin per particle (412) and is the expected result based on a one to one binding ratio. In addition to rat IgG2b, we have routinely added anti-mouse CD11b, anti-mouse CD11c, anti-mouse CD80, and armenian hamster IgG isotype antibodies to PRINT particles (see **Figure 9**).

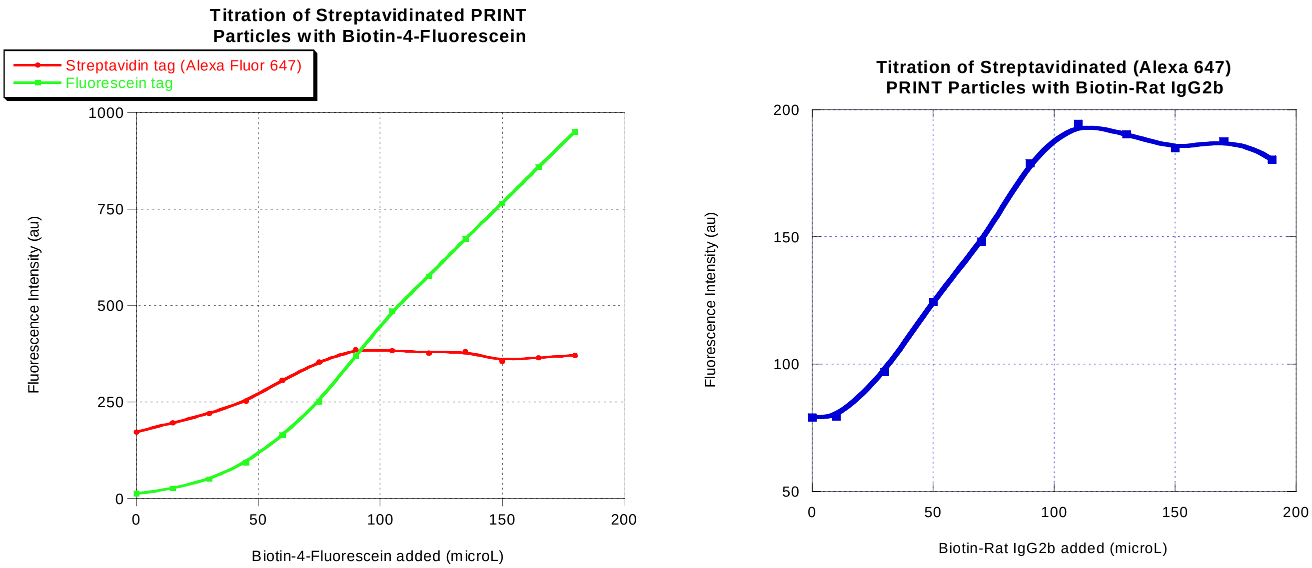


Figure 8 – Determination of surface streptavidin (Alexa Fluor 647) concentration on PRINT particles by titration with biotin-4-fluorescein (left), and determination of surface biotin-rat IgG2b isotype control antibody concentration on PRINT particles by titration with biotin-rat IgG2b isotype control (right).

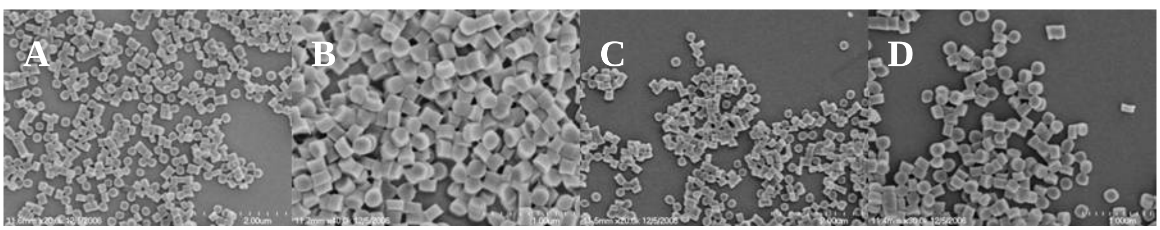


Figure 9 – SEM images of cylindrical PRINT particles (200 nm diameter × 200 nm height) that have anti-mouse CD11b (A), anti-mouse CD11c (B), anti-mouse CD80 (C), and rat IgG2b (D) conjugated to the surface.

The initial concentration of coupled avidin was too low to allow effective binding of PRINT to professional APC. As a result we needed to alter the coupling ratios to increase the number of avidin molecule/PRINT. This has allowed effective targeting. **Figure 10** (left) shows the targeting efficacy obtained. While we have been successful in increasing the specific binding, we believe we can still improve the sensitivity by higher coupling efficiencies.

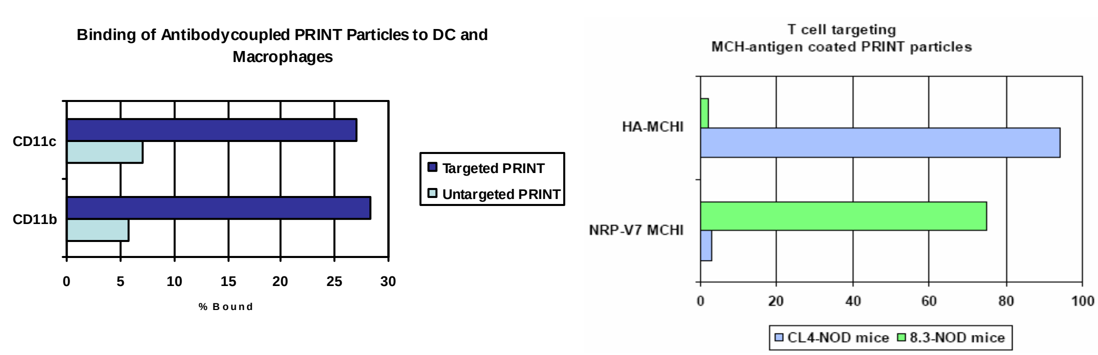


Figure 10: Left: Selective targeting of PRINT particles to either CD11b or CD11c expressing antigen presenting cells in vitro. **Right**: Particles decorated with MHC/peptide complexes and their selective targeting to T cells.

Increasing the coupling concentration even by a factor of 2 might help. Decorating the outside of the same particles with MHC/peptide molecules also results in extremely selective uptake by antigen specific T cells. In the 8.3-NOD mice, 75% of their CD8+ T cells are transgenic and will recognize the NRP-V7 MHCI (Kd) tetramer. In the CL4-NOD mice, almost all of their CD8+ T cells are transgenic and will recognize the HA-MHCI (Kd) tetramer. We found that the NRP-V7-Kd coated PRINT particles targeted approximately 75% of the CD8+ T cells in the spleen of the 8.3-NOD mice but only 3.74% of the CD8+ T cells in the spleen of the CL4-NOD mice. The HA-Kd coated PRINT particles targeted 94% of the CD8+ T cells in the spleen of the HA-NOD mice but only 1% of the CD8+ T cells in the spleen of the 8.3-NOD mice.

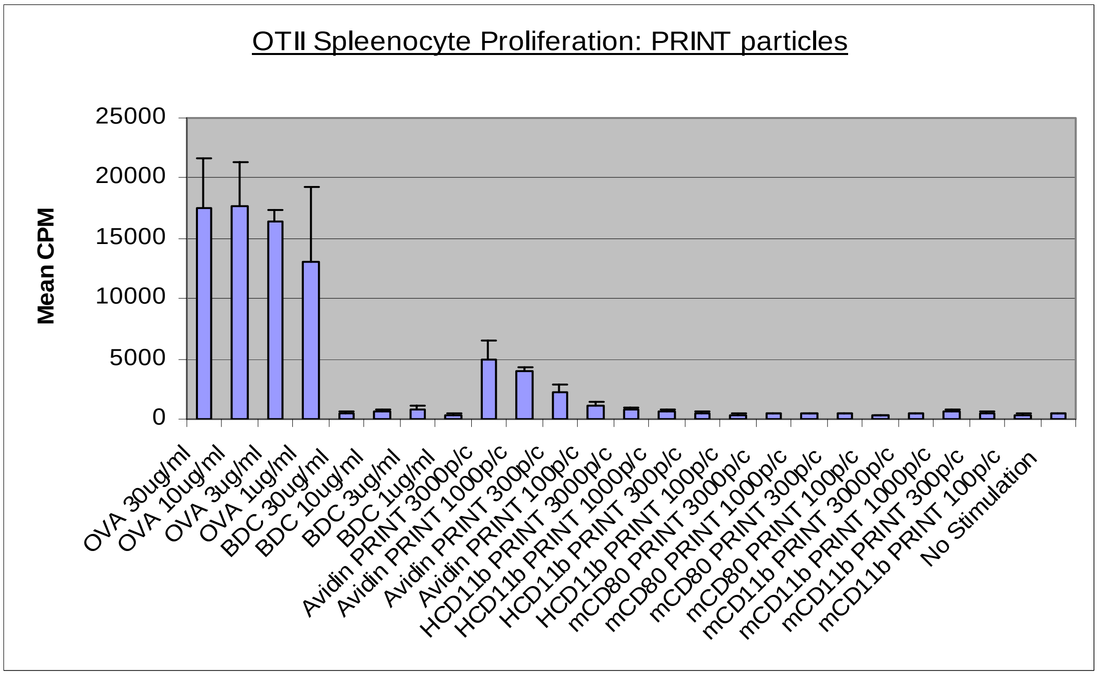


Figure 11. OVA=a peptide from ovalbumin. These cells are transgenic for a T cell receptor that recognizes ova, so these are our positive control samples.
BDC=a peptide from a diabetes antigen called BDC2.5. These cells should not recognize this antigen, so these are our negative control samples.

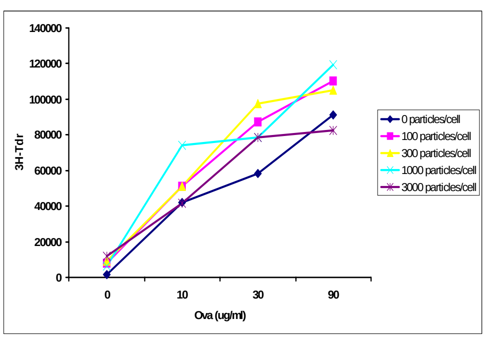


Figure 12. PRINT particles do not interfere with the proliferative response of CD4 T cells to ovalbumin.

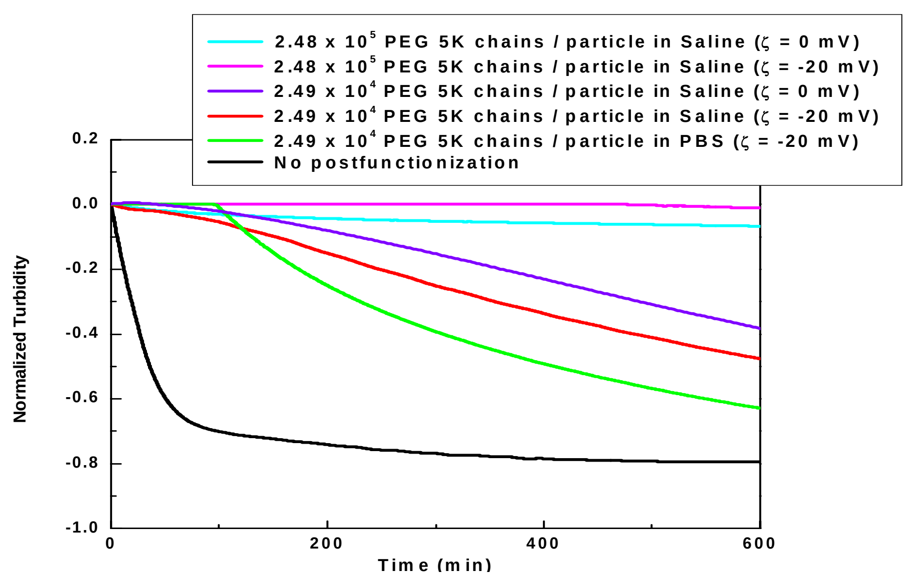
As shown in **Figure 11**, the CD80-PRINT and CD11b-PRINT particles themselves did not stimulate any proliferative response. The PRINT particles that had no antibody on them (e.g. the Avidin only PRINT particles) seem to be slightly stimulatory for splenocyte proliferation. This is likely due to the nonspecific stickiness of avidin for receptors on the surface of the T cells, leading to crosslinking of unknown stimulatory receptors. When the antibodies to CD80 and CD11b were on the beads, they probably blocked access to the avidin. The strong response to the avidinated particles was a little surprising. The non-specific binding interactions were greatly attenuated in the antibody-coated particles.

We have also made certain that the PRINT particles themselves have no nonspecific impact on immune responses in vitro. We have titrated the immune response to ovalbumin in vitro. In this experiment we performed titrations of ova, in the presence of different levels of PRINT particles. As seen in **Figure 12**, we see no evidence of inhibition of the ova response, using proliferation of OT-II T cells as readout. Exposure to up to 3000 PRINT particles per cell has no effect on the ability of T cells to recognize ovalbumin. Ovalbumin loaded as a cargo in PRINT nanoparticles can be used to stimulate OT1 and OTII T cells *in vitro*.

To successfully accomplish milestone 3, both specific targeting and cargo release need to fully optimized and characterized using an in vitro system. There are several goals that needed to be accomplished before moving onto the in vivo experiments for milestone 3. The first is to increase in vivo circulation half life. A number of PEG-based nanoparticle compositions have been examined to find the optimal composition for increased particle stability in solution and longer circulation times in vivo. UV-vis turbidity measurements were used to assess particle stability in aqueous solutions at a concentration of 3.2 mg/mL, which is the dosing concentration currently used for the in vivo biodistribution studies. The most stable particle compositions studied to date are PEG-based systems, functionalized after fabrication with 5K PEG. Two ligand densities were investigated, 2.5×10^4 and 2.5×10^5 (estimated number of PEG chains/ 200 nm nanoparticle). The two densities show solution stability in both PBS and saline for a minimum of several hours with the particles having the highest density of PEG chains (Figure 13 - pink and light blue curves) showing the greatest stability. The stability observed for these particle compositions represents an order of magnitude improvement compared to the nanoparticles used in prior biodistribution studies (black curve). For the nanoparticles used in previous studies, the PEG chains were incorporated into the PRINTed polymer solution instead of post functionalized. Preliminary biodistribution results for the 5K PEG nanoparticles suggest that we are seeing a factor of 4 increase in the circulation half-life when compared to previous results.

We would also like to optimize targeting and cargo release. While we have been successful in observing specific binding, we believe we can still improve the specificity by higher coupling efficiencies. Increasing the coupling concentration even by a factor of 2 might help. Work is on-going in this area in combination with the particle stabilization efforts described above. Characterization of the disulfide based particles is also moving ahead. An understanding of the release profiles under varied conditions, the loading and additional in vitro studies are all on-going.

Figure 13: Plot of turbidity as a function of time. Turbidity used to assess stability of an aqueous solution of particles.



Milestone #4

To assess T cell responses *in vitro*, bone marrow derived dendritic cells will be treated with PRINT particles. By using targeted particles, we expect that we will be able to induce proliferation of $CD4^+$ and $CD8^+$ transgenic T cells.

Jeff Frelinger and Joseph DeSimone

We have demonstrated our ability to target dendritic cells in bulk splenocytes in vitro using anti-CD11b, anti-CD11c, and anti-CD80 as targeting ligands. With those results in hand we focused on delivering two ova-peptide fragments to dendritic cells both in vitro and in vivo to stimulate T cell responses. We have chosen to test one ova-peptide fragment that is Class I restricted ($OVA_{257\text{-}264}$ – SIINFEKL).

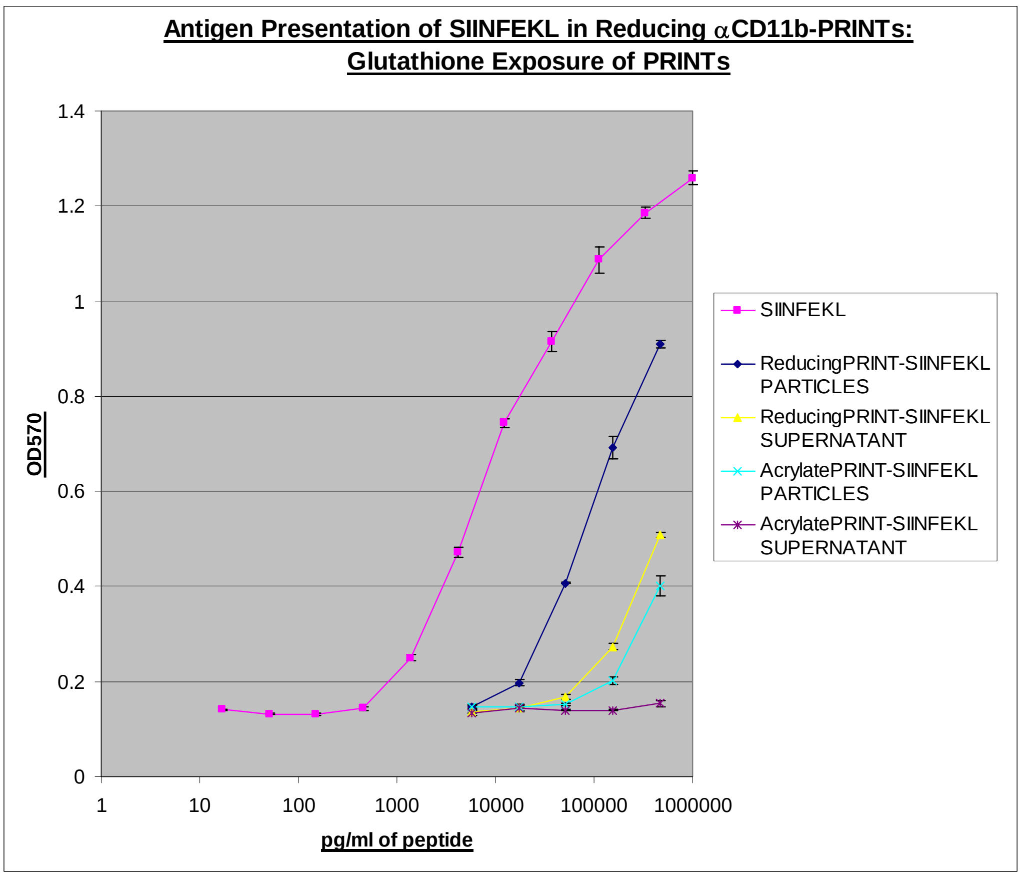


Figure 14 - T-cell response to Ova-peptide loaded PRINT particles. The readout for antigen presentation is observed by measuring CPRG absorbance (570nm). Free ova peptide, SIINFEKL is shown in pink. The response for the disulfide-based nanoparticles is as follows: released peptide, SUPERNATANT is shown in yellow and the particle-retained peptide PARTICLES is shown in dark blue. The response for the acrylate-based is as follows: released peptide, SUPERNATANT is shown in purple and the particle-retained peptide PARTICLES is shown in light blue.

The composition of the 1 µm × 1 µm cylindrical PRINT nanoparticles used in this experiment was PEG_{900}-triacryalte (67 wt%), AEM (10 wt%), N,N'-cystaminebisacrylamide (20 wt%), ova peptide (2 wt%), HCPK (1 wt%). The PRINT particles were reduced by incubating overnight at 4°C in a 100 mM solution of glutathione in phosphate buffered saline. Spleen cells were isolated from a four week old C57B/6. PRINT particles were pretreated overnight with 100 mM glutathione to reduce the particles. Released peptide (SUPERNATANT) and particle-retained peptide (PARTICLES) were titrated on spleenocytes and incubated at 37°C overnight followed by B3Z incubation also at 37°C overnight. CPRG in Z-Buffer (100 mM beta-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40, in PBS) was added, reacted for 4 hours to lyse the cells to release the β-galactosidase enzyme generated by the NFAT-LacZ construct. B3Z cells are Ova peptide (SIINFEKL) responsive MHC I restricted hybridoma T-cells that contain a reporter construct that induces LacZ driven by an NFAT responsive promoter, and respond to antigen presentation by increasing NFAT transcription, thus inducing LacZ. The readout for antigen presentation is observed by measuring CPRG absorbance (570nm) and shows that ova-peptide loaded disulfide-PRINT particles successfully delivered Ova peptide to antigen presenting cells, in a bulk spleenocyte pool, for the proper presentation of this antigen to MHC class I specific Ova responsive T-cells (**Figure 14**). It is also clear from the results obtained that the activated release (disulfide-based particles) was significantly more effective than passive release (acrylate-based particles).

<u>Milestone #5</u>

We would like to add back chemical functionality by linking specific viral surface functionality, such as E. coli produced hepatitis B surface antigen, to the surface of shape specific PRINT particles. Again, this will be a test that demonstrates the ability of antigen-linked PRINT particles to evoke an immune response that is similar to natural infection or vaccination. For further proof we will complete a PMBC experiment. In this case we would use HepB vaccinated human donor cells and particles analogous to those in milestone three, with anti human CD205 as the targeting ligand on the outside and HepB surface antigen as the cargo. Again, we would measure T cell cytokine to determine the efficacy of the recall immune response.

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A major objective of the proposed research was to generate PRINT nanoparticles that mimic viral surface for applications in both gene delivery and immunology. We have now identified and developed three components of AAV that can be exploited for such purposes. **First,** several different serotypes of AAV have been developed into vectors. Each serotype has a unique capsid topology and displays unique tissue tropisms and immune profile. A particularly attractive feature of each AAV serotype is its ability to evade neutralizing antibodies against other serotypes. Each serotype will serve as a template for exploring the potential of generating PRINT particles that might mimic AAV serotypes or display new immune profiles. We have also developed a novel mutant that contains minimal changes at the amino acid level and displays a new immune profile. Through this process, we have identified the two-fold axis of symmetry referred to as the canyon region as an important antigenic site. These initial studies suggest that the 2-fold dimple region on AAV2 and possibly other serotypes is a "hot spot" for antibody neutralization and could well play a critical role in determining the tropisms of several AAV serotypes. More importantly, the reproduction of such antigenic regions on PRINT nanoparticles could generate surfaces with novel immunological profiles or those that might mimic differences between AAVserotypes.

A **second** aspect of AAV vectors that can be exploited in the context of AAV vectors is the targeting capabilities of the capsid. To this effect, we have generated biotinylated AAV capsids using sulfo-NHSLC-biotin (Pierce) for attachment to PRINT particles labeled with streptavidin molecules. In addition, the AAV capsid also contains a phospholipase A2 domain that cleaves membrane lipids enabling endosomal escape and nuclear entry. We have now generated phospholipase A2 domains derived from AAV capsids that can be chemically or endogenously biotinylated. These 18kDa protein moieties will also be tested for their ability to promote intracellular delivery of PRINT nanoparticles by conjugation to streptavidin coated surfaces.

Lastly, we have developed a new strategy to produce and purify AAV genomes with the inverted terminal repeats necessary for long term persistence. Entrapping these novel constructs in PRINT nanoparticles will help overcome the packaging limitation of AAV vectors and for allow long term gene expression in various tissues. To this effect, we have developed a system for large scale production of single-stranded AAV genomes as well as double-stranded vectors.

Milestone #6

In order to demonstrate that molecular imprinting can be combined with PRINT, we will first show that "viral recognition surfaces" can be made on thin films. We will begin by using microcontact printing to form a patterned self-assembled monolayer (SAM) on a gold substrate containing our ligand of interest and a hydrophobic moiety. Then, we will introduce a complimentary ligand to the monolayer. After verification that our complimentary functionality has associated with the ligand of interest, we will add PFPE and cure. Careful analysis by XPS and Chemical AFM will be completed to first prove the association has occurred and then to verify that the complimentary functionality has been incorporated in a specific geometry into the PFPE mold.

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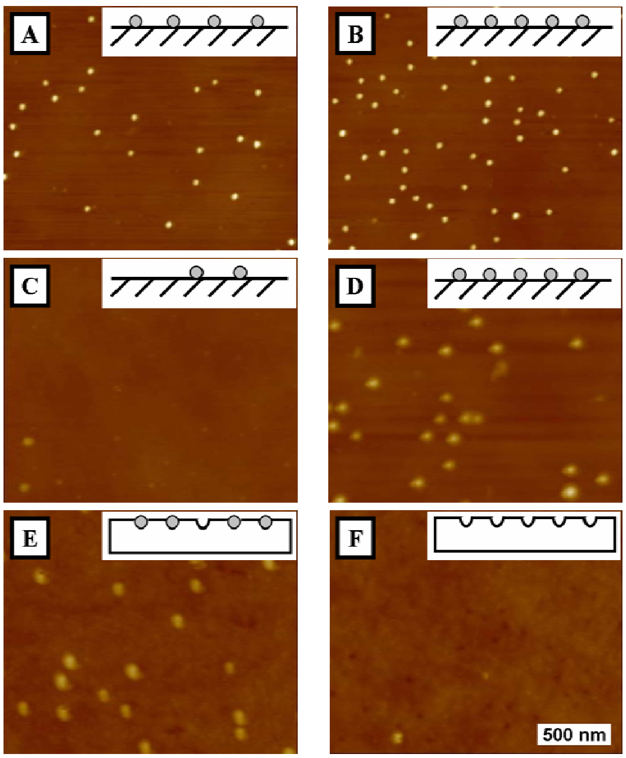

Figure 15. AFM images of PS-b-PI spherical micelles on modified silicon (A) $\gamma_A = 51$ mJ/m^2 and (B) $\gamma_B = 57$ mJ/m^2 before molding. Micelle coverage on (C) $\gamma_A = 51$ mJ/m^2 and (D) $\gamma_B = 57$ mJ/m^2 after molding. PFPE molds from (E) $\gamma_A = 51$ mJ/m^2 and (F) $\gamma_B = 57$ mJ/m^2 after lift-off.

Preparation of viral replicas by PRINT is controlled by the physical interaction between viral particles (viruses) and the substrate. The interaction depends on the chemical composition of both the particles and the substrate. The interaction also depends on the shape of particles (e.g. globular and rod-like viruses). In order to understand the interplay between the chemical composition and shape, we studied replication of model systems: spherical, cylindrical, and torroidal block-copolymer micelles. One of the main advantages of the PRINT technology is its ability to mold freely-lying and weakly-adhering particles. This process depends on (i) particle/substrate interaction and (ii) particle geometry. When working with freely-lying particles, two scenarios can occur: either the particles remain on the substrate, or the particles can be lifted-off from the substrate and remain on the PFPE mold (**Figure 15**).

We proposed using polystyrene-b-polyisoprene (PS-b-PI) micelles as model nanometer-sized objects to study molding and harvesting of particles adsorbed to a solid substrate. The specific objectives are (i) to measure the minimal surface energy of the substrate that allows molding and (ii) to elucidate the effect of particle geometry on the molding process.

- We prepared a series of substrates with different surface energies to determine the critical substrate surface energy for the particle lift-off.
- For spherical micelles, a critical surface energy was determined to be 54±3 mN/m. For surface energies below 54 mN/m, almost all micelles were lifted from the substrate (**Figure 15**a,c,e). For higher surface energies, the micelles remain adsorbed to the substrate resulting in a PFPE mold with well-defined spherical cavities that can be used for further replication (**Figure 15**b,d,f).
- In the case of lower surface energies (below 54 mN/m), we demonstrated reversible transfer of particles from the PFPE mold (after lift-off, Fig.1e) to a new substrate with a higher surface energy.
- Currently, we study molding of PS-b-PI particles of different geometries, such as cylinders and torroids. The goal is to determine the effect of geometry on the critical surface energy**.**

Building upon the work described above molding self-assembled molecular aggregates, the objective going forward will be to make a mold of a protein antigen or an antibody and then make a PRINT nanostructure replicating the topological structure of the biological entity. The biological activity of the protein mimic can them be tested.

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